

## Cadmium stimulates MAPKs and Hsp27 phosphorylation in bovine adrenal chromaffin cells

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### Abstract

Cadmium ( $\text{Cd}^{2+}$ ) is a common environmental pollutant, which is widely used in industry and is a constituent of tobacco smoke. Exposure to this heavy metal has been linked to a wide range of detrimental effects on mammalian cells. In this study, the action of  $\text{Cd}^{2+}$  on protein phosphorylation in bovine adrenal chromaffin cells (BACCs) was examined. Cells were incubated with  $^{32}\text{P}$ i in the presence of  $\text{Cd}^{2+}$  (1–50  $\mu\text{M}$ ) and proteins were separated by one- or two-dimensional electrophoresis. An increase in the phosphorylation of BACCs proteins, without changing cell viability, was observed in response to  $\text{Cd}^{2+}$  (5–50  $\mu\text{M}$ ). Particularly at three spots, with molecular weight of 25 kDa and isoelectric point range 4.0–4.5, which were identified as phosphorylated isoforms of the heat shock protein of 27 kDa (Hsp27). Phosphorylation of the  $\text{p38}^{\text{MAPK}}$ , a member of mitogen-activated protein kinase (MAPK) family, was stimulated by  $\text{Cd}^{2+}$  over the same concentration range and it was the major upstream protein kinase involved in the phosphorylation of all three spots of Hsp27.  $\text{Cd}^{2+}$  also stimulated the phosphorylation of other MAPK family member, the extracellular signal-regulated kinase (ERK)-1/2. Therefore, primary adrenal chromaffin cells are a target for  $\text{Cd}^{2+}$  and both the ERK1/2 and the  $\text{p38}^{\text{MAPK}}$  are activated. Additionally, Hsp27 is highly phosphorylated in response to the metal exposure, due to  $\text{p38}^{\text{MAPK}}$  activation. These biochemical effects of  $\text{Cd}^{2+}$  might disrupt the normal secretory function of these cells.

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### 1. Introduction

Cadmium ( $\text{Cd}^{2+}$ ) is a ubiquitous industrial and environmental pollutant that accumulates in humans and

animals (Waalkes, 2003; Waisberg et al., 2003). Exposure to cadmium occurs through intake of contaminated food or water, or by inhalation of tobacco smoke or polluted air (Waalkes, 2003; Nawrot et al., 2006). The tissues that accumulate  $\text{Cd}^{2+}$  include the kidneys (Madden and Fowler, 2000), lung (Waalkes, 2003; Nawrot et al., 2006), reproductive organs (Takiguchi and Yoshihara, 2006) and nervous system (Yoshida, 2001; Lukawski

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et al., 2005).  $\text{Cd}^{2+}$  causes genotoxicity and cancer in some tissues and nonmalignant chronic toxicity in others (Madden and Fowler, 2000; Filipic et al., 2006). For example, in the lungs  $\text{Cd}^{2+}$  is linked to cancer (Nawrot et al., 2006), in the kidneys long term accumulation of  $\text{Cd}^{2+}$  causes mainly nephrotoxicity (Madden and Fowler, 2000) and in the nervous and endocrine systems  $\text{Cd}^{2+}$  disrupts secretion and action of neurotransmitters and hormones, including effects on memory formation (Sorimachi et al., 1999; Yoshida, 2001; Henson and Chedrese, 2004; Lukawski et al., 2005; Takiguchi and Yoshihara, 2006).

The cellular actions of  $\text{Cd}^{2+}$ , such as cell death, carcinogenesis and disruption of neurotransmitter and hormone action, are extensively documented, but the molecular mechanisms underlying these actions are still not resolved (Waalkes, 2003; Waisberg et al., 2003; Hirano et al., 2005). It is established that  $\text{Cd}^{2+}$  has pleiotropic effects including the induction of immediate early genes (i.g. c-fos, c-jun) and genes encoding for metallothioneins and heat shock proteins (Hsps) (Waisberg et al., 2003), induction of oxidative stress (Waisberg et al., 2003; Filipic et al., 2006) and interference with calcium signaling (Beyersmann and Hechtenberg, 1997; Misra et al., 2002).

Mitogen-activated protein kinases (MAPKs) are a family of highly conserved enzymes, which comprise ubiquitous groups of signaling proteins that play critical regulatory roles in cell physiology (Chang and Karin, 2001; Chen et al., 2001). Members of each major MAPK subfamily, the extracellular signal-regulated protein kinases (ERK1/2), c-Jun N-terminal kinases (JNK) and  $\text{p38}^{\text{MAPK}}$ , have been implicated in the modulation of metabolism, neurotransmission, cell survival or death and cell proliferation or differentiation (Chen et al., 2001; Park et al., 2006). These MAPKs are also likely to be involved in the molecular mechanisms of  $\text{Cd}^{2+}$ 's action since it has been reported that activation of ERK1/2, JNK and  $\text{p38}^{\text{MAPK}}$  occurs in renal cells (mesangiais or glomerular) (Ding and Templeton, 2000; Hirano et al., 2005), in macrophages (Radloff et al., 1998; Misra et al., 2002) and tumoral cell lineages (Hung et al., 1998; Galán et al., 2000; Son et al., 2001; Lee et al., 2005).

Heat shock proteins are a family of stress-inducible proteins that show a functional heat shock element in their promoter. Within the cell they can form large oligomers, which serve as chaperones that bind other proteins and regulate their conformation and activity (Sharp et al., 1999). Mammalian Hsp27 is rapidly phosphorylated in multiple sites (Landry et al., 1992) by MAP kinase-activated protein kinase-2 (MK2) in

response to various extracellular stresses and the activation of MK2 is dependent on  $\text{p38}^{\text{MAPK}}$  (Stokoe et al., 1992; Freshney et al., 1994; Rouse et al., 1994). Non-phosphorylated Hsp27 is an oligomeric protein that works as chaperone and confers resistance against oxidative stress (Preville et al., 1998; Rogalla et al., 1999). On the other hand, phosphorylation of Hsp27 disaggregates oligomeric complexes leading to Hsp27 dimers or tetramers (Kato et al., 1994), an event that has been suggested as essential for protection against heat shock and oxidative stress (Huot et al., 1996; Geum et al., 2002) and which leads to association of Hsp27 with microfilaments thereby regulating their dynamics (Lavoie et al., 1993; Benndorf et al., 1994; Landry and Huot, 1999). Phosphorylation of Hsp27 via activation of  $\text{p38}^{\text{MAPK}}$  has been reported in macrophages and mesangial cells in response to  $\text{Cd}^{2+}$  (Radloff et al., 1998; Hirano et al., 2005) and in response to  $\text{Pb}^{2+}$  in adrenal chromaffin cells (Leal et al., 2002). In human glioma cells,  $\text{Cd}^{2+}$  induces rapid dissociation of the aggregated form of Hsp27 by phosphorylation, which may be involved in a cellular defense mechanism for protection against stress (Kato et al., 1994). Additionally, the exposure to heavy metals such as  $\text{Cd}^{2+}$  may induce the expression of heat shock proteins such as Hsp27 and Hsp70 (Bonham et al., 2003; Croute et al., 2005).

Adrenal chromaffin cells are responsible for the synthesis and secretion of adrenaline and noradrenaline into the blood. It has been reported that exposure to  $\text{Cd}^{2+}$  increases intracellular  $\text{Ca}^{2+}$  via generation of 1,4,5-IP<sub>3</sub> in cultured bovine adrenal chromaffin cells (BACC) (Yamagami et al., 1998). In addition, in cat chromaffin cells  $\text{Cd}^{2+}$  at micromolar concentration stimulates catecholamine secretion via  $\text{Ca}^{2+}$  (Yamagami et al., 1994; Sorimachi et al., 1999).

The ERK1/2 and  $\text{p38}^{\text{MAPK}}$  pathways play fundamental roles in chromaffin cells, especially regulating the neurosecretory machinery (Ait-Ali et al., 2004; Park et al., 2006) and the activity of tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of the catecholamines (Bobrovskaya et al., 2001, 2004; Dunkley et al., 2004). BACCs are extensively studied cells that represent an excellent model to perform basic studies of cell signaling pathways (Thomas et al., 1997; Dunkley et al., 2004). It is important determine the possible molecular effects of metal contaminants such as  $\text{Cd}^{2+}$  upon signal transduction mechanisms in these cells. The aim of the present study was to determine in BACCs the effects of  $\text{Cd}^{2+}$  on the MAPKs (ERK1/2 and  $\text{p38}^{\text{MAPK}}$ ) and on the phosphorylation of the Hsp27 a recognized endpoint of the  $\text{p38}^{\text{MAPK}}$  pathway.

## 2. Materials and methods

### 2.1. Chemicals

Anti-phospho-p38<sup>MAPK</sup> antibody and anti-Hsp27 antibody were from Calbiochem. SB203580, bovine serum albumin (BSA), HEPES and anti-phospho-ERK1/2 antibody were from Sigma. Cadmium chloride was from BDH. [<sup>32</sup>P]orthophosphate (<sup>32</sup>P<sub>i</sub>; carrier free; 10 mCi/ml) was from Du Pont NEM. Acrylamide/bis-acrylamide and NBT/BCIP were from Bio-Rad. Hybond nitrocellulose (ECL), secondary antibodies (rabbit and mouse) HRP-conjugated and ECL detection reagents were from Amersham Bioscience. Sequence grade trypsin was from Promega. All other reagents were of analytical grade, or tissue culture grade for cell culture.

### 2.2. Cell cultures

BACCs were isolated from adrenal glands by pronase and collagenase digestion followed by Percoll density gradient centrifugation, as described by Bunn et al., 1995. Cells were then plated onto 6-well culture plates in a supplemented DMEM medium (Bunn et al., 1995) at a density of  $2 \times 10^6$  cells per well and maintained at 37 °C. Cultured cells were used for experimentation between the 3rd and 12th days.

### 2.3. Cell treatment and <sup>32</sup>P<sub>i</sub> labeling

BACC were removed from the incubator, placed on a warming plate held at 37 °C. Cells were washed twice with 1 ml of HEPES-buffered saline (HBS) containing 10 mM HEPES, 154 mM NaCl, 5.2 mM KCl, 1.2 mM MgCl<sub>2</sub>, 1.2 mM CaCl<sub>2</sub>, 6 mM glucose, pH 7.4. Incubation was then performed for 1 h with a further 1 ml of the same medium containing <sup>32</sup>P<sub>i</sub> 50 μCi/ml. Cadmium chloride was dissolved in double deionized water before use. The metal (1–50 μM) was added to the incubation medium containing <sup>32</sup>P<sub>i</sub> and incubation performed for 1 h. The p38<sup>MAPK</sup> inhibitor, SB203580 (1 μM), was added 15 min before the incubation with the metal.

### 2.4. Cell viability

Following Cd<sup>2+</sup> treatment, BACC were incubated with MTT (5 mg/ml in PBS), for 45 min at 37 °C. In viable cells MTT is converted into a purple formazan dye after cleavage of the tetrazolium ring by mitochondrial dehydrogenases. Formazan was dissolved by incubating cells with DMSO for 30 min at 37 °C. The assay was quantified by an ELISA reader (550 nm) (Mosmann, 1983).

### 2.5. One-dimensional gel electrophoresis

The reactions were stopped with 300 μl/well of SDS-stopping solution (4% SDS, 2 mM EDTA, 8% β-Mercaptoethanol, 50 mM Tris, pH 6.8). The cells were scraped from each well and solubilized. Samples were fractionated by

SDS-PAGE using gradient gels 7.5–15% or 12% gels (Bunn et al., 1995). When the phosphoproteins were <sup>32</sup>P<sub>i</sub> radiolabeled, 40 μg protein/track was applied and they were detected by autoradiography (Dunkley et al., 1997). While, for phosphorylation measurement through Western blot, 60 μg protein/track was applied for phospho-ERK1/2 immunodetection, and 120 μg protein/track for phospho-p38<sup>MAPK</sup> immunodetection.

### 2.6. Two-dimensional electrophoresis

In order to analyse the proteins by two dimensional gel electrophoresis (2D), the reactions were stopped with 10% TCA (4 °C). Cells were scraped and centrifuged (4 °C) at  $15,000 \times g$  for 5 min. The pellet was washed twice with 4% TCA and once with ethanol 80%. The final pellet was resuspended in 300 μl of Rehydration Solution (7 M urea, 2 M Thiourea, 4% CHAPS, 2% Ampholines, 40 mM Tris, 0.3% Dithiothreitol, bromophenol blue). The sample (3 mg protein) was applied to IPG strips by in-gel hydration. IPG strips (Pharmacia, pH 4–7, 13 cm) were rehydrated for at least 6 h in the Rehydration Solution to their original thickness of 0.5 mm. The strips were then focused in the first dimension on a Pharmacia LKB Multiphor system. The gel strips were run at 300 V for 5 h, then increased to 3500 V over 5 h, before continuing at 3500 V for 6 h. Before the second dimension was run strips were equilibrated in Equilibration solution (6 M Urea, 20% glycerol, 2% SDS, 375 mM Tris pH 8.8 plus 1% DTT or 4% iodoacetamide). IPG strips were placed in the top of 1 mm thick, 12% SDS-PAGE gels and held in place by 1% agarose. The gels were then run at 25 mA/gel until the blue dye front had reached the bottom of the gel (approximately 5 h). Proteins were electrotransferred to PVDF membrane using 10 mM Caps buffer with 10% methanol at 600 mA overnight and stained with Coomassie Blue. The membranes were exposed to X-ray films and the phosphoproteins were identified on the autoradiograms.

### 2.7. Immunoblotting

BACC proteins separated by one-dimensional SDS-PAGE were transferred to nitrocellulose (Jarvie and Dunkley, 1995; Leal et al., 2002). The membranes were blocked with 2% BSA in TBS-T (0.1% Tween 20 (v/v), 25 mM Tris, 150 mM NaCl, pH 7.5). In BACC samples phospho-p38<sup>MAPK</sup> was detected with anti-phospho-p38<sup>MAPK</sup> (1:1000; incubated overnight); phospho-ERK1/2 was detected with anti-phospho-ERK1/2 (1:10,000; incubated 2 h), all antibodies were diluted in TBS-T (150 mM NaCl, 10 mM Tris, pH 7.6, 0.05% Tween-20) containing 2% BSA. After extensive washing using TBS-T, the membranes were incubated 1.5 h with secondary antibody. Either anti-rabbit IgG-HRP (horseradish peroxidase) or anti-mouse IgG-HRP (1:4000 in 1% BSA/TBS-T) was used as a secondary antibody. The reactions were developed by ECL. Hsp27 was identified on PVDF, after separation of proteins by 2D electrophoresis, using the anti-Hsp27 (rabbit antibody (1:2000 in 2% BSA/TBS-T). The reaction was determined by a colorimetric assay using anti-rabbit IgG, conjugated to alkaline

phosphatase and nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrate.

## 2.8. Quantification and statistic analysis

Protein phosphorylation was measured by scanning the autoradiograms and the bands were quantified using the Analyst program from Biorad. Data are presented as mean  $\pm$  S.D. or  $\pm$  S.E. Statistical significance was assessed by one-way ANOVA followed by Duncan's test, when appropriate. A value of  $p < 0.05$  was considered to be significant.

## 3. Results

Incubation of BACC with cadmium ( $\text{Cd}^{2+}$ ; 5–50  $\mu\text{M}$ ) increased the phosphorylation of  $\text{p38}^{\text{MAPK}}$  and ERK1/2 (Fig. 1). There was also an increase in the phosphorylation of other BACC proteins exposed to the same concentrations of  $\text{Cd}^{2+}$  and analysed by  $^{32}\text{P}$ i labeling (Fig. 2A). In particular there was a significant increase in the  $^{32}\text{P}$ i incorporation into a protein of 25 kDa (pp25 kDa; Fig. 2A and B). This increase in phosphorylation of pp25 kDa was blocked by 1  $\mu\text{M}$  SB203580 (Fig. 2C), a classic inhibitor of  $\text{p38}^{\text{MAPK}}$ .

$\text{Cd}^{2+}$  can induce toxic effects on cells (Galán et al., 2000; Watjen et al., 2002). However, there were no significant effects on the viability of BACC after exposure to 1–50  $\mu\text{M}$   $\text{Cd}^{2+}$  for 1 h (Fig. 3).

Analyses of the 25 kDa band by two-dimensional electrophoresis showed that in fact  $\text{Cd}^{2+}$  caused an increase in the  $^{32}\text{P}$ i incorporation into a number of spots, but especially into three spots (spots 1–3; Fig. 4A versus C). Phospho-spot 1, the more acidic, only appears in the presence of  $\text{Cd}^{2+}$  (Fig. 4C), while phospho-spots 2 and 3 are both seen under basal conditions. SB203580 substantially blocked the effects of  $\text{Cd}^{2+}$  (50  $\mu\text{M}$ ) on all three spots (Fig. 4D). Spots 1–3 were identified as phosphorylated forms of Hsp27 using an Hsp27 antibody and the fourth spot (spot 4) as an unphosphorylated form of Hsp27 (Fig. 5). Sequence analysis of two tryptic peptides derived from spot 3 determined their sequences to be VSLDVNHFAPEE and SATQSAEITIPV (Leal et al., 2002). A BLASTP search of the SWISSPROT database confirmed that these sequences uniquely matched with 27 kD heat shock protein (Hsp27).

## 4. Discussion

The present study shows that  $\text{Cd}^{2+}$ , in a short incubation period of 1 h, activates the  $\text{p38}^{\text{MAPK}}$ /Hsp27 phosphorylating system in BACCs, without changing the cell viability. Additionally, it shows that ERK1 and

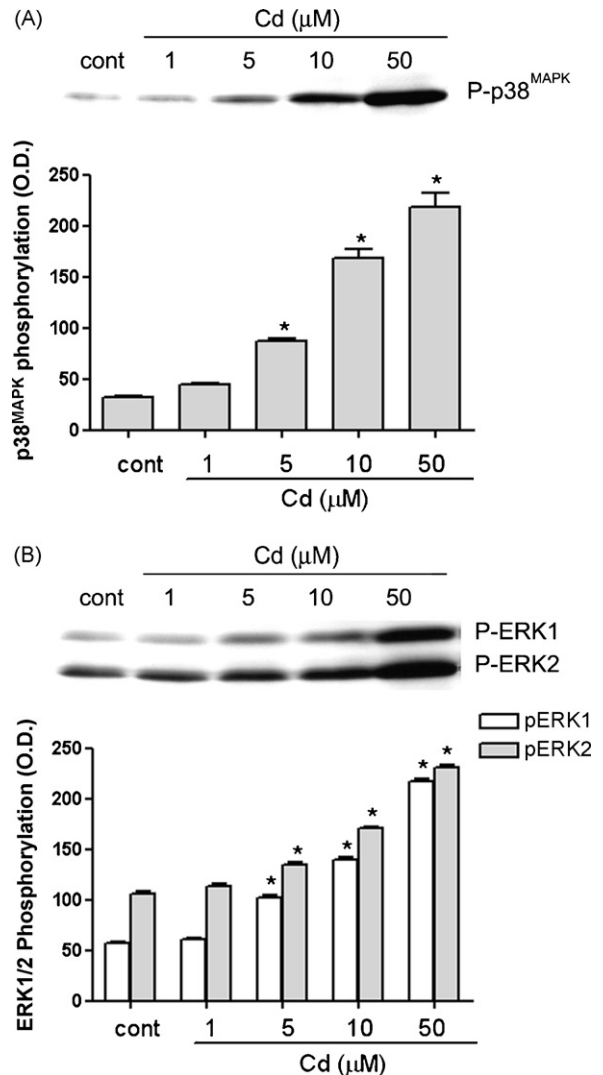
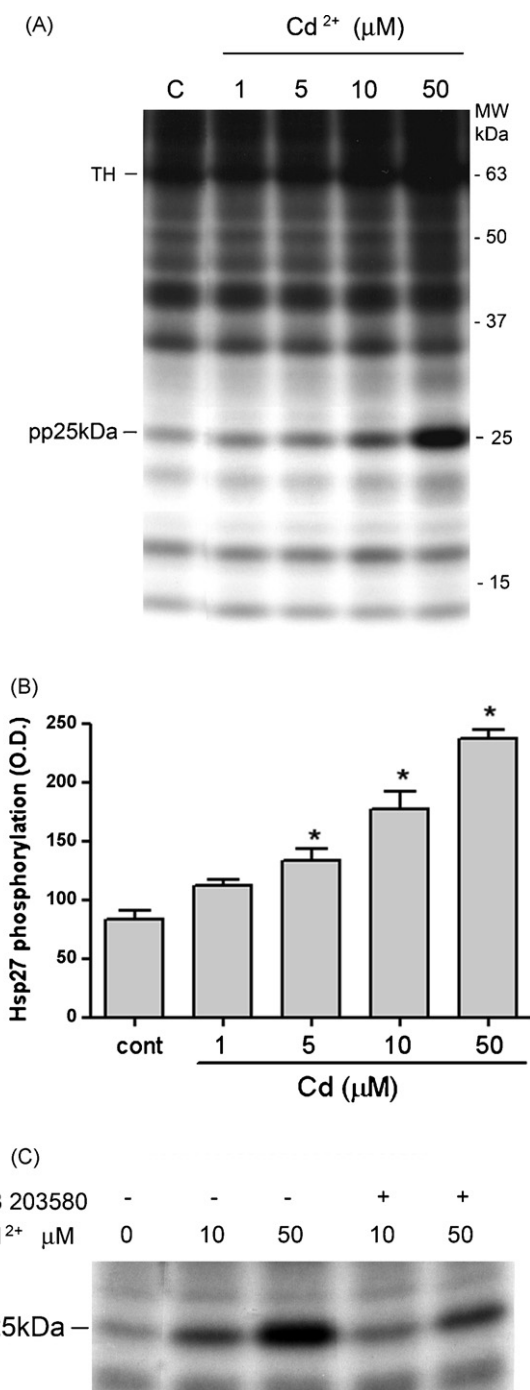


Fig. 1. Modulation of  $\text{p38}^{\text{MAPK}}$  and ERK1/2 phosphorylation by  $\text{Cd}^{2+}$ . BACC were incubated 1 h in presence of various concentrations of  $\text{Cd}^{2+}$ . Cells were disrupted using SDS and proteins were separated by one-dimensional electrophoresis and transferred to a nitrocellulose membrane. Phosphorylation of  $\text{p38}^{\text{MAPK}}$  (A) and ERK1/2 (B) were detected by specific antibodies against the bi-phosphorylated region of each kinase and reactions were developed by ECL. Phosphorylation was quantified by densitometric analysis. The data are expressed as percentage of the control (considered as 100%). The values are a mean of 3–4 experiments  $\pm$  S.D. Statistical significance, \* $p < 0.001$  compared to control. Phospho- $\text{p38}^{\text{MAPK}}$  (P- $\text{p38}^{\text{MAPK}}$ ) and phospho-ERK1/2 (P-ERK1 and P-ERK2) show differences between treatments ( $p < 0.01$ ).

ERK2 are strongly activated by  $\text{Cd}^{2+}$ . Lead ( $\text{Pb}^{2+}$ ) also causes activation of  $\text{p38}^{\text{MAPK}}$  leading to Hsp27 phosphorylation (Leal et al., 2002) in BACCs. However,  $\text{Pb}^{2+}$  was not able to activate ERK1/2 in these cells. Therefore,  $\text{Cd}^{2+}$  and  $\text{Pb}^{2+}$  act on different MAPK pathways in BACCs.



The activation of p38<sup>MAPK</sup> by Cd<sup>2+</sup> and the consequential phosphorylation of Hsp27 has been shown in neuroblastoma lineage cells (Lee et al., 2005), in human glioma cells (Kato et al., 1994), in rat renal cells (mesangial and glomerular cells) (Hirano et al., 2005), in rat sertoli cells (Pittenger et al., 1992) and in bovine alveolar macrophages (Radloff et al., 1998).



In the present study, BACCs stimulated by Cd<sup>2+</sup> showed three major phosphorylated forms of Hsp27 when analysed by 2D (Figs. 4 and 5). These forms represent different states of phosphorylation of different sites on the protein. Our data are consistent with the p38<sup>MAPK</sup> activation by Cd<sup>2+</sup> and as a consequence activation of MAP kinase-activated protein kinase-2 (MK2) (Thomas et al., 1997; Bobrovskaya et al., 2001; Leal et al., 2002), which is recognized as a major kinase acting on Hsp27 phosphorylation (Sharp et al., 1999; Vertii et al., 2006; Zheng et al., 2006). ERK1/2 phosphorylation and activation by Cd<sup>2+</sup> did not lead to Hsp27 phosphorylation in BACC as PD98059 (20 μM), which inhibited ERK1/2 phosphorylation (Bobrovskaya et al., 2001), had no effect on Hsp27 phosphorylation (data not shown).

Human Hsp27 has three phosphorylation sites for MK2: Ser15, Ser78 and Ser82 (Landry et al., 1992; Stokoe et al., 1992; Rogalla et al., 1999; Butt et al., 2001). In addition, it has been demonstrated that phosphorylation of Thr143 on Hsp27 may occur by activating protein kinase G (PKG) in human platelets (Butt et al., 2001). On the other hand, in rodents only two phosphorylation sites have been observed (Ser15 and Ser86) (Gaestel et al., 1991; Stokoe et al., 1992). Analyses by 2D gel electrophoresis of Cd<sup>2+</sup>-stimulated sertoli cells and mesangial cells lineages (both from rat) showed only two phosphorylated Hsp27 spots (Pittenger et al., 1992; Hirano et al., 2005). Therefore, our data showing three phosphorylated spots after bovine chromaffin cells were exposed to Cd<sup>2+</sup> is closer to the effects seen in human cells than in rodent cells (Gaestel et al., 1991; Landry et al., 1992). The sequence analyses of bovine Hsp27 (Q3T149; <http://www.ebi.uniprot.org>) shows high homology with human Hsp27 (P04792; <http://www.ebi.uniprot.org>), for each phosphorylating

Fig. 2. Cd<sup>2+</sup> increases the phosphorylation of BACC proteins including a 25 kDa band. BACCs were incubated with <sup>32</sup>P<sub>i</sub> for 1 h in the presence or absence of Cd<sup>2+</sup>. Cells were disrupted using SDS and proteins were separated by one-dimensional SDS-PAGE. (A) Representative autoradiograph showing the effect of Cd<sup>2+</sup> on the overall phosphorylation pattern of BACC proteins. (B) Effects of Cd<sup>2+</sup> on pp25 phosphorylation. The quantification of the pp25 band was performed by densitometric analysis and the data are expressed as percentage of the control (considered 100%). The values are a mean of 4 independent experiments ± S.D. Statistic significance, \**p* < 0.001 compared to control. There was difference between the treatments (*p* < 0.01). (C) Representative Western blotting showing that the selective p38<sup>MAPK</sup> inhibitor SB203580 (1 μM) blocked the Cd<sup>2+</sup>-induced phosphorylation of pp25. BACCs were pre-incubated 15 min with 1 μM SB203580, the p38<sup>MAPK</sup> inhibitor was maintained throughout the incubation in the continuing presence of Cd<sup>2+</sup> and <sup>32</sup>P<sub>i</sub>. As control, cells were <sup>32</sup>P<sub>i</sub> labeled in presence or absence of Cd<sup>2+</sup> (10 and 50 μM), but without addition of inhibitor.

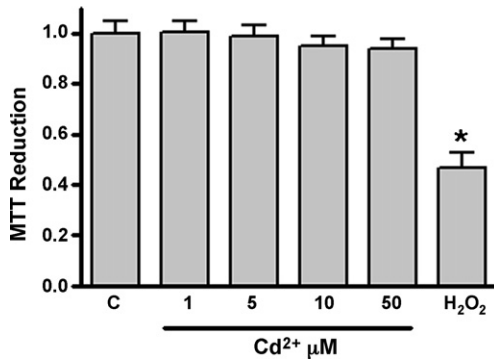


Fig. 3. Viability of BACC after exposure to Cd<sup>2+</sup>. BACC were incubated for 1 h in presence of concentrations of Cd<sup>2+</sup> from 1 to 50 µM. Cell viability was analysed by measurement of MTT reduction. H<sub>2</sub>O<sub>2</sub> (1 mM) was used as a positive control for the MTT assay. The values are a mean of 16–32 experiments ± S.E. Statistical significance, \**p* < 0.01 compared to controls.

site previously identified in human (Landry et al., 1992; Butt et al., 2001) (Fig. 6). We showed that the three Hsp27 phosphorylated spots were substantially inhibited by low concentrations (1 µM) of SB203580, indicating a clear dependence on p38<sup>MAPK</sup> activation in order to phosphorylate the diverse sites on bovine Hsp27. SB203580 is a highly selective inhibitor of p38<sup>MAPK</sup> α and β isoforms, and the inhibition of other kinases such as Raf-1, tyrosine kinases and GSK3 only occurs at 5–100-fold higher concentrations (Davies et al., 2000;

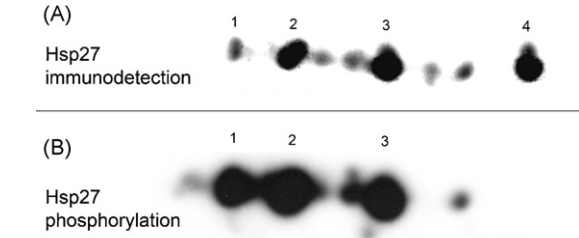


Fig. 5. Analysis of Hsp27 isoforms after exposition of BACCs to Cd<sup>2+</sup>. Cells were incubated for 1 h with Cd<sup>2+</sup> (50 µM). Proteins were separated by 2D PAGE and transferred to PVDF as described in Fig. 3. The figure shows part of the membrane containing the 25 kDa region. (A) Immunodetection of Hsp27 performed with anti-Hsp27 antibody. (B) Autoradiogram of the spots 1–3 shown in Fig. 3C (Cd<sup>2+</sup>). Numbers 1–4 indicate the spots that consistently reacted with the antibody.

English and Cobb, 2002). However, we cannot rule out the possible participation of additional protein kinases. This might include PKG (Butt et al., 2001), which can be activated by Cd<sup>2+</sup> (Watjen et al., 2001), or AKT that in human neutrophils phosphorylates Hsp27 and form a complex with the protein together to p38<sup>MAPK</sup> and MK2 (Rane et al., 2003; Zheng et al., 2006).

What are the pathophysiological consequences of Hsp27 phosphorylation in BACCs? Hsp27, a member of the small heat shock proteins, is a powerful ATP-independent chaperone that may participate in cellular processes, including the suppression of protein aggrega-

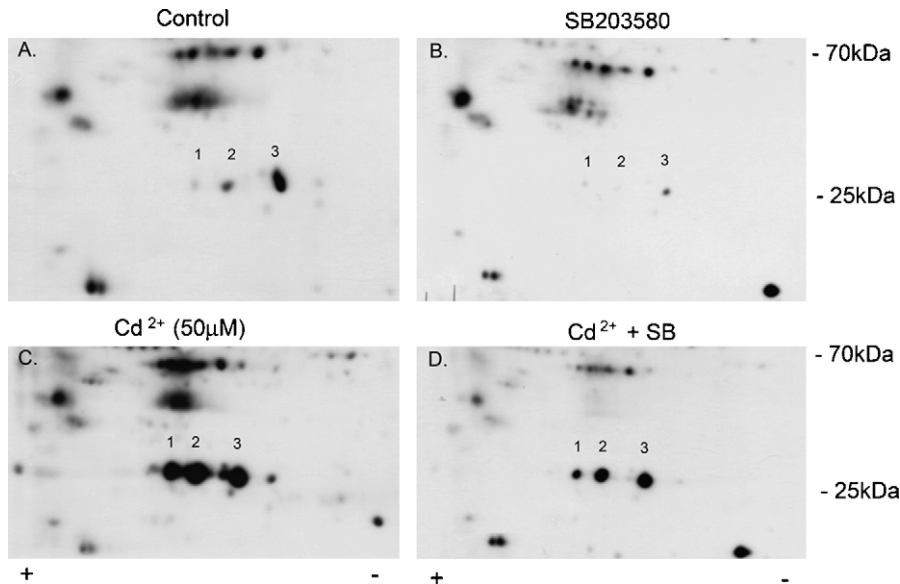


Fig. 4. Modulation of protein phosphorylation by Cd<sup>2+</sup> analysed by two-dimensional electrophoresis. BACC were labeled in the same conditions described at Fig. 2. However, for these studies the proteins were precipitated with TCA and solubilized in Rehydration Solution containing urea, thiourea and CHAPS, and then separated by 2D electrophoresis. (A) Control; (B) SB203580, 1 µM; (C) Cd<sup>2+</sup>, 50 µM; (D) Cd<sup>2+</sup>, 50 µM plus SB203580, 1 µM. Cd<sup>2+</sup> caused a consistent increase on the level of phosphorylation of three phosphoproteins (spots 1–3) and the effect was blocked by the selective p38<sup>MAPK</sup> inhibitor SB203580.

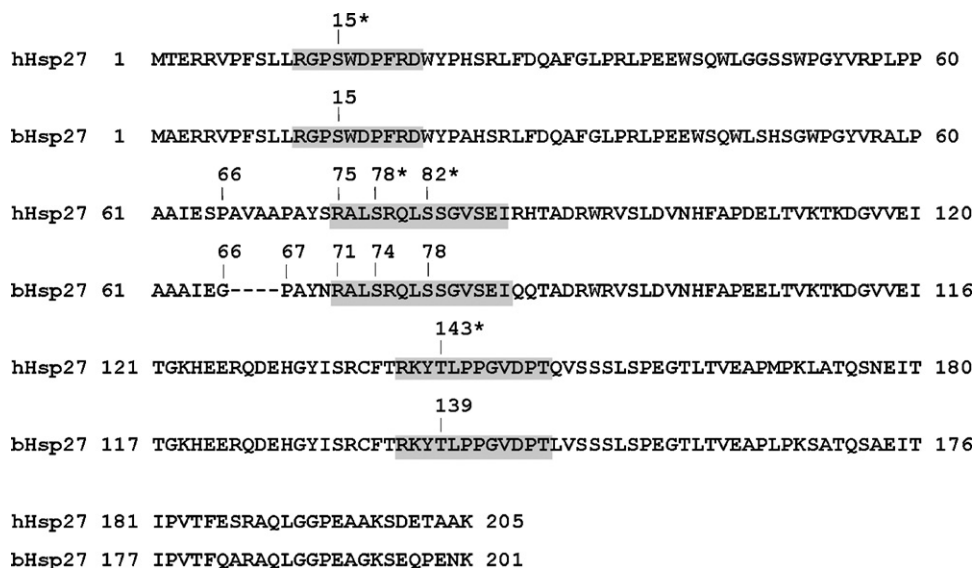


Fig. 6. Comparison between amino acid sequence of human Hsp27 (hHsp27) and bovine Hsp27 (bHsp27). The amino acid sequence of hHsp27 (P04792) and bHsp27 (Q3T149) proteins were obtained from the <http://www.ebi.uniprot.org>. Each phosphorylating site previously identified on hHsp27 (Landry et al., 1992; Rogalla et al., 1999; Butt et al., 2001) are indicated by the asterisk and include: Ser 15, Ser 78, Ser 82 and Thr 143. The bHsp27 sequence was placed in order to indicate the high homology with the human protein. The putative phosphorylating sites on bHsp27 (Ser 15, Ser 74, Ser 78 and Thr 139) and the described phosphorylated sites on hHsp27 are highlighted.

tion, dynamics of cytoskeletal proteins and, cell growth, differentiation and death (Mounier and Arrigo, 2002; Parcellier et al., 2003; Didelot et al., 2006). The function of Hsp27 phosphorylation is still enigmatic. However, it is generally agreed that phosphorylation modulates Hsp27 function by changing its oligomerization, sub-cellular localization and/or protein–protein interactions (Kato et al., 1994; Sharp et al., 1999; Vertii et al., 2006). After phosphorylation Hsp27 decreases its oligomeric size, leading to a loss of both its chaperone activity and its ability to protect against oxidative stress (Rogalla et al., 1999; Vertii et al., 2006). On the other hand, it has been pointed that Hsp27 phosphorylation confers stabilization of actin filaments, which might result in cell protection (Lavoie et al., 1993; Benndorf et al., 1994; Kato et al., 1994; Mounier and Arrigo, 2002; Valentim et al., 2003). Therefore, activation of the  $p38^{\text{MAPK}}$  pathway in response to  $\text{Cd}^{2+}$ , followed by phosphorylation of Hsp27, may lead, on the one hand, to a decrease in its chaperone and antioxidant actions, and on the other hand, to a modification of the polymerization state of Hsp27 with increasing actin filament stability.

The activation of ERK1/2 by  $\text{Cd}^{2+}$  has also been well documented in a diversity of cell models (Hung et al., 1998; Ding and Templeton, 2000; Galán et al., 2000; Hirano et al., 2005). We have shown that it is the same for BACCs. Since, ERK1/2 modulates various biochemical

steps in BACCs such as phosphorylation of TH (Dunkley et al., 2004), the proteins of exocytotic machinery (Ait-Ali et al., 2004; Park et al., 2006), and modulates the  $\text{Na}_v1.7$  sodium channel  $\alpha$ -subunit (Yanagita et al., 2003). It is possible that disturbing MAPK phosphorylation by  $\text{Cd}^{2+}$  may also interfere with the catecholamine production and secretion, as well as BACCs excitability. The molecular disorder caused by  $\text{Cd}^{2+}$  on adrenal chromaffin cells, especially the alterations of signal transduction pathways, has not been extensively studied. However, the functional consequences of cat and bovine adrenal chromaffin cells acutely exposed to  $\text{Cd}^{2+}$  at micromolar range include an increase at cytosolic  $\text{Ca}^{2+}$  concentration with simultaneous stimulation of catecholamine secretion (Yamagami et al., 1994, 1998; Sorimachi et al., 1999) by mechanisms that, may involve production of inositol 1,4,5-triphosphate (Yamagami et al., 1998), e/or induction of membrane depolarization due to the decrease in  $\text{K}^+$  conductance (Sorimachi et al., 1999).

The present study did not address to the mechanisms for activation of ERK1/2 or  $p38^{\text{MAPK}}$ . Nevertheless, based on the general effects of  $\text{Cd}^{2+}$  on mammalian cells and the known regulation of MAPK pathways, it may involve production of reactive oxygen species (ROS) (Waisberg et al., 2003; Leonard et al., 2004; Wang et al., 2004); inhibition of tyrosine phosphatases (Tonks, 2005; Samet et al., 1999; Tal et al., 2006), or be a conse-

quence of an increase of intracellular  $\text{Ca}^{2+}$  (Yamagami et al., 1998; Sorimachi et al., 1999; Waisberg et al., 2003; West et al., 2002; Agell et al., 2002).

Regardless of the molecular mechanisms of MAPK activation, our findings contribute by indicating that  $\text{Cd}^{2+}$  induces activation of ERK1/2 and the  $\text{p38}^{\text{MAPK}}$ . Furthermore, we demonstrated, in BACCs, that Hsp27 a specific endpoint for the  $\text{p38}^{\text{MAPK}}$  pathway (Bobrovskaya et al., 2001; Leal et al., 2002) is highly phosphorylated in response to  $\text{Cd}^{2+}$ . These biochemical effects of  $\text{Cd}^{2+}$  might disrupt the normal secretory function of these cells. Additionally, these cell signaling targets for  $\text{Cd}^{2+}$  may be important in the brain and other endocrine tissues, where  $\text{Cd}^{2+}$  exposure alters neurotransmitter release and neural plasticity.

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